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(71) Applicant *(for all designated States except US)*: INSTITUTO NACIONAL DE ENGENHARIA E TECNOLOGIA INDUSTRIAL/DEPARTAMENTO DE TECNOLOGIA DE INDÚSTRIAS QUÍMICAS [PT/PT]; Azinhaga dos Lameiros, P-1699 Lisboa (PT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : MEIRINHOS DA CRUZ, Maria, Eugénia [PT/PT]; Rua Maestro Raul Ferrão, 43, P-1500 Lisboa (PT). ATAÍDE DE CARVALHOSA NUNES DIAS, Isabel, Maria, Costa, Mimoso [PT/PT]; Rua Rui de Mascarenhas, Lote 7, R/C Esq., Vila Fria, P-2780 Oeiras (PT). GAMEIRO FRANCISCO, Ana, Paula [PT/PT]; Praceta Maria Lamas, Lote 43, 3° Esq., Olival de Basto, P-2675 Odivelas (PT).

(74) Agent: DE ARANTES E OLIVEIRA, João; Rua do Patrocínio, 94, P-1300 Lisboa (PT).

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(57) Abstract

The invention refers to liposomal formulations containing an antibiotic, comprising drug/lipid ratios up to 10 mg/100 mg lipid, the size of liposomes ranging from 5 μm to 0.01 μm and the encapsulation efficiencies typically being greater than 60 %. The liposomal antibiotics, when administered to animals, increase the half-life circulation time in plasma, efficiently reduce the acute toxicity and increase the antimicrobial activities, compared to the free drugs. The invention also refers to a process for the preparation of liposomal formulations comprising forming multilamellar liposomes containing the antibiotic and subjecting the liposomes to lyophilization, rehydration and optionally extrusion under pressure.

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DESCRIPTION

"LIPOSOMAL AMINOGLYCOSIDE COMPOSITIONS AND PROCESS FOR THEIR PREPARATION"

Background of the invention

This invention relates to liposomal preparations containing relatively high levels of charged molecules, such as phosphatidylinositol, resulting in increased entrapment efficiency of aminoglycoside drugs and increased therapeutic effectiveness and lower toxicity.

The aminoglycosides are a family of bactericidal antibiotics which contain amino sugars in glycosidic linkages. They are polycations and their polarity is primarily responsible for the pharmacokinetic properties shared by the group. The aminoglycosides inhibit protein synthesis in a variety of microorganisms, and are useful therapeutically and prophylactically in the treatment of serious, often life-threatening bacterial infections. They are particularly useful in the treatment of infections (e.g., septicemia, peritonitis, pneumonia, urinary tract infections) due to organisms which are resistant to other antibiotics, such as species of Pseudomonas, E. coli, Klebsiella, Enterobacter, Salmonella, Listeria, Mycobacteria, Staphylococcus, etc. (Soares et al., Farmácia Portuguesa, 34:23-41 (1985); Wright et al., J. Chem. Soc. Chem. Commun., 6:206 (1976); Miller et al., Antimicrob. Agent. Chemother., 10:827-836 (1976); Sande et al., "As Bases Farmacológicas da Terapêutica", Vol. II, pp. 1018-1034, ed. Guanabara Koogan S.A. (1983); and Luft, J. Int. Med. Res., 6:286-299 (1978).

The use of aminoglycosides is often limited by

potentially serious adverse toxicities. Among these are ototoxicity, nephrotoxicity and a potentially fatal neuromuscular paralysis. The ototoxicity effects can involve both cochlear (auditory) and vestibular toxicity. The nephrotoxicity effects include abnormalities of tubular resorption and renal morphology. The muscular paralysis and difficulty in respiration may be due to blockage of the neuromuscular junction due to the aminoglycosides by inhibiting acetylcholine release. The order of increasing ability of the aminoglycosides to effect acute toxic reactions often correlates with the ability to effect nephrotoxicity.

The mechanism underlying the toxicities of the aminoglycosides may be associated with the ability of the aminoglycosides to bind to polyphosphoinositides found in inner ear and Kidney tissues. It has been postulated that phosphatidylinositol diphosphate serves as a receptor for the drugs and renders these tissues more sensitive than others to this family of drugs. Lodhi et al., Biochem. Pharmacol. 29:597-601 (1980). Thus, considerable effort has focused on these putative aminoglycoside receptors as a means to ameliorate the associated toxicities.

In one approach it has been proposed that aminoglycosides could be complexed with phosphatidylinositolphosphates prior to administration to reduce interactions between the drug and the endogenous toxicity receptor. See Janoff et al., U.S. Patent No. 4,897,384. Although Janoff et al. also discuss the possibility of administering these drug-lipid complexes as liposomes, the difficulty remains in formulating stable liposomes with charged lipids such as phosphatidylinositol in the presence of water, as would be necessary for most aminoglycosides. Further, in Janoff the drug portion of the drug-lipid conjugate is exposed to the patient and may become separated from the conjugate in the patient.

Liposomal formulations of aminoglycosides have

been suggested as a way to achieve higher potency pharmaceutical preparations with smaller volume and thus causing less tissue injury upon administration. Liposomes have shown evidence of protection against the toxicity effects. (Guargon et al., Antimicrob. Agents Chemother. 34:235-240 (1990); Micier et al., Antimicrob. Agents Chemother. 34:343-348 (1990); and Bally et al., WO 88/04573 (1987)). However, these liposome-aminoglycoside formulations are obtained with high drug/lipid ratios and have low encapsulation efficiencies. Low encapsulation efficiencies result in increased use of drug in the preparatory process and a resulting increase in cost. For example, in Bally et al., the phosphate salts of aminoglycoside were proposed to overcome a limited association of aminoglycosides with liposome preparations. The drug:lipid ratio was approximately 1:1 to 1:3, and encapsulation efficiency was not more than 40%.

The encapsulation of proteins and later aminoglycosides into liposome formulations was also discussed in Cruz et al., Liposomes in the Therapy of Infectious Diseases and Cancer, pp. 417-426 1989 Liss, Inc., NY, and Mimoso et al., Congresso Nacional de Biotecnologia, 1990, Braga, Portugal. The total lipid concentration was relatively low, 16 μmol/ml, and a low relative concentration of phosphatidylinositol (10%) was used. The final compositions having a drug:lipid ratio of 2-4 μg/μmol.

In Francisco et al., Third Congresso Nacional de Ciências Farmacêuticas, 1991, Lisbon, Portugal, formulations of liposome and aminoglycoside were reported to have an encapsulation efficiency of 25-30% and a drug/lipid ratios of 10-15 μg/μmol, and conferred an increased drug half-life and survival rate in treated animals, but no explanation was provided as to the formulations and methods used to prepare the drug/liposome compositions.

Accordingly, what is needed in the art is a method for preparing liposomal formulations of aminoglycosides with high incorporation efficiency, low relative toxicity to the patient, and high pharmacological activity against the microorganism. Quite surprisingly, the present invention fulfills these and other related needs.

Summary of the Invention

The present invention relates to liposomal formulations containing an antibiotic, characterized by having drug/lipid ratios up to 10 mg/100 mg lipid, the size of liposomes ranging from 5 μm to 0.01 μm and the encapsulation efficiencies typically being greater than 60%. The liposomal antibiotics, when administered to animals, increase the half-life circulation time in plasma, efficiently reduce the acute toxicity and increase the antimicrobial activities, compared to the free drugs.

The process for the preparation of liposomal formulations is characterized by forming multilamellar liposomes containing the antibiotic and subjecting the liposomes to lyophilization, rehydration and optionally extrusion under pressure.

Description of the Specific Embodiments

The processes of the present invention provide liposomal formulations of aminoglycosides with high incorporation efficiency, low toxicity and high pharmacological activity. The present invention comprises a process of dehydration and rehydration, followed by an optional extrusion process through a porous membrane or

other liposome sizing procedure. This process comprises the following main steps: a) multilamellar liposomes (MLV) are prepared which contain intra and extra-liposomal drug; b) using dextrose or other physiologic osmolarity medium in the rehydration step; c) avoiding a freeze-thaw step before lyophilization; d) using a high dilution volume of liposomes before optional filtration; e) using as starting material for extrusion liposomes which have not been previously separated from extraliposomal drug.

According to the present invention, to prepare the multilamellar liposomes a chloroform lipid mixture (ranging from about 10 mg lipid/ml chloroform up to 100 mg/ml or more) is dried under nitrogen stream. The resulting lipid concentration ranges from about 30 mM up to about 120 mM, preferably about 45 to 75 mM, and in certain preferred embodiments described in the Examples below range from 49 mM up to about 61 mM. Suitable lipids, hydrogenated or not, for use in the formulations present individually or in mixtures, in a molar ratio ranging from 0.01 to 10: cholesterol (Chol), phosphatidylcholine (PC), distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimirystoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatidic acid (PA), dicetylphosphate (DcP), dimirystoylphosphatidylglycerol (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and any similar synthetic lipids. The lipid mix is preferably negatively charged, and most preferably containing a relatively high concentration of phosphatidylinositol, hydrogenated phosphatidylinositol, phosphatidylinositol mono and biphosphate and the like. The liposomal preparations are typically mixtures of at least two components and more usually three or more: a glycerophospholipid (e.g.,

phosphatidylcholine, dimirystoylphosphatidycholine, dipalmitoylphosphatidylcholine); cholesterol (optionally present); and a negatively charged molecule (lipidic or not) such as phosphatidylinositol, dicetylphosphate, with each component of the liposomal preparation (when present) in molar ratios of 40-70%, 10-30%, and 20-50%, respectively. For example, a preferred combination is phosphatidylcholine: cholesterol: phosphatidylinositol at 5:1:4, with total lipid concentration ranging from 49 to about 64 mM. The components of the lipid mixture are chosen so at least one component, e.g., phosphatidylinositol, is capable of binding the aminoglycoside antibiotic and is present in the lipid mixture at a concentration of at least about 20-60%, preferably at least about 25% to 40% concentration of total lipid components. The resultant negatively charged lipid starting mix produces higher antibiotic encapsulation efficiencies and stable liposomes while minimizing drug-associated toxicities and maximizing antibiotic effectiveness.

The lipidic film is hydrated with an aqueous solution of antibiotic, such as an aminoglycoside. The concentration of the drug solution can vary considerably, from as low as about 0.05 mg/ml up to as much as 100 mg/ml or more, but more typically 1 mg/ml up to 10 mg/ml, e.g., 4.6 mg/ml as described in an Example below. The corresponding concentration of lipid for hydration ranges up to 50 mg/ml to as high as 100 mg/ml or more. It is desirable to produce liposomes with high drug/lipid ratio, without exceding saturation of lipidic membrane of liposomes. Going beyond saturation the encapsulation efficiency will be reduce without increasing intraliposomal drug. Thus, it is important that the encapsulation efficiency of the process to be as high as possible to produce liposomes containing adequate amounts of therapeutic drug, even for small initial amount of drug.

The antibiotic is preferably an aminoglycoside,

such as neomycin, Kanamycin, amikacin, tobramycin, gentamicin (including gentamicin C_1, C_{1a}, and C_2), sisomicin, netilmicin, streptomycin, paromomycin and other members of the aminoglycoside family as described in, e.g., Drug Evaluations, Chpt. 6, Amer. Med. Assn. 1990, but other antibiotics (e.g., polyene antibiotics and non-antibiotic drugs may also benefit from the processes of the present invention. In Examples described below a particularly preferred aminoglycoside is netilmicin. As noted above, the concentration of the aminoglycoside in the aqueous solution used to hydrate the lipid film and thus in the final liposome can vary considerably, depending on the particular drug chosen, the components of the lipidic mixture, the ionic strength, osmolarity, pH, temperature and incorporation method used. The antibiotic or derivatives in the resulting liposome/antibiotic formulation can be contained primarily in the aqueous phase, in the lipid phase, or in the aqueous and lipid phase.

The multilmellar liposomes so formed are then subjected to freezing either in liquid nitrogen (-170°C) or for one hour in a deep freezer (-70°C) followed by lyophilization on a freeze dryer (lyophilizer) at 25 mtorr for overnight. The resulting powder is rehydrated in two successive steps: first with a sugar solution at a portion (e.g. 1/10) of the initial volume of physiologic osmolarity, e.g., a 5% solution of dextrose, galactose, mannose, sucrose, etc. that does not interfere with drug activity with vigorous vortexing followed by a stabilizing rest period at room temperature of about 30 min.; secondly the volume is brought up to the initial volume with saline solution (e.g., NaCl 154 mM) followed by a 10 min. stabilizing period at room temperature. The resulting liposome/drug mixture is then diluted 5- to 10-fold with saline solution and, optionally, is sized. Finally the sized or non-sized mixture is ultracentrifuged at 250,000 g during 30 min. and the pellet resuspended in saline

solution.

Several techniques are available for sizing liposomes to a desired size. One sizing method is homogenization, which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0,1 and 0,5 microns, are observed. Extrusion of liposome through a small-pore polycarbonate membrane ou an asymmetric ceramic membrane under pressure is also an effective method for reducing liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposomes size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes (e.g., from 3.0 μm down to 0.01 μm, as desired) to achieve a gradual reduction in liposome size. In any of the methods the particle size distribution can be monitored by conventional laser-beam particle size discrimination.

A particularly preferred embodiment of the invention produces liposome/aminoglycoside formulations with high encapsulation efficiency at a low initial drug:lipid ratio. In this embodiment the lipid mixture is phosphatidylcholine: cholesterol: phosphatidylinositol at 5:1:4, with relative lipid concentration of 49-64 mM. Hydration takes place with, e.g., 2.3 to 4.6 mg/ml netilmicin, followed by freezing at -170°C or -70°C and lyophilization. Rehydration to a final osmolarity of 300 mOSM is accomplished by the addition of 5% dextrose in one-tenth the volume of antibiotic used, followed by filling to the initial volume with 154 mM NaCl. Upon dilution in saline and ultracentrifugation as described above, the final liposomal/aminoglycoside formulation has a diameter of about 0.900 μm ± 0.095 μm, but can be smaller if extruded, and possessed an encapsulation efficiency of 66 ±

1%, a drug:lipid ratio of 1:30 to 1:40 and a drug: phosphatidylinositol ratio of 1:12-1:15. As with the other preparations described herein the drug is secluded from the external medium (in contrast to typical drug-lipid conjugates) and cannot be displaced from the liposome by a stronger ligand as might occur in other preparations. The improved therapeutic and pharmacokinetic effects that result are not necessarily related to the final amount of drug in the liposome/drug mixture, but the fact that the drug is in a liposomal form having the characteristics described herein.

The present process for aminoglycoside encapsulation is a significant improvement over earlier protocols, e.g., as decribed in Bally et al., WO 88/04573 (1987), for a number of reasons which include, among others: (1) the present process starts from multilamellar liposomes containing drug and not from empty liposomes to which drug is later added; (2) the process does not use the combination of freeze-thaw steps before lyophilization; (3) the process produces liposomes having a physiological osmolarity, using dextrose or the like in rehydration step, which is preferred for pharmaceutical administration; (4) the problem of forming stable aminoglycoside:liposome formulations using a relatively high concentration of a negatively charged lipid such as phosphatidylinositol is solved; and (5) low starting concentrations of aminoglycoside still permit high encapsulation efficiencies.

The liposomal/aminoglycoside formulations prepared according to the processes of the present invention exhibit encapsulation efficiencies greater then 60% without alteration of <u>in vitro</u> biological activity. Moreover, when injected in animals (mice) a liposomal/aminoglycoside formulation prepared as described herein demonstrated a circulating time 12-18 fold longer when compared to the free (non liposomal) aminoglycoside

(netilmicin). The present formulations also evidenced a reduced toxicity compared to free drug, wich is particularly significant in view of the toxicities typically associated with aminoglycosides and the fact that their use is severely limited by those toxicities. In vivo toxicity studies in animals (mice) showed that lethal dose 50 (LD_{50}) of liposomal netilmicin formulations is higher (at least 50 mg/kg) than the LD_{50} of the free form (20-24 mg/kg). Accordingly, the liposomal formulations of aminoglycosides produced according to the present invention may be administered at higher concentrations, and hence enhanced efficacy, without fear of increased toxicity to a patient.

The high encapsulation efficiencies achieved with low drug/lipid ratios indicate that the liposomal/aminoglycoside formulations of the invention are also highly effective as therapeutic agents. In an in vivo model of peritonitis infection caused by an aminoglycoside-sensitive bacterial suspension of E. coli, the formulations prepared by the present process demonstrated a higher therapeutic activity when compared to free aminoglycoside. The results appear better than the other published liposomal aminoglycoside formulations, since the present formulations have high encapsulation efficiencies of at least about 60%, preferably as high as 85 to 90%, with low initial drug/lipid ratios (0.47-5.6 mg / 100 mg lipid) (49 ± 11 μg/μmol and 34 ± 2 μg/μmol). These results also indicate that the liposomal/aminoglycoside formulations described here (particularly those which employ netilmicin) are highly effective agents in the prophylactic or therapeutic treatment of serious infections caused by gram-negative bacteria. The prolonged action of liposomal/netilmicin also permits a reduction in frequency of drug administration and monitoring of drug concentration and the medical care associated therewith. The larger size liposomes, those which are not extruded and range in size

from 0.8-1 μm, may be more efficacious when used therapeutically than the smaller, extruded liposomes (0.1-0.2 μm) which have longer residence times in the circulation and thus are particularly useful when administered prophylactically.

The liposomal/antibiotic formulations of the invention may contain additional substances which serve to target the liposome and hence antibiotic to a particular tissue or cell, such as a bacterial, as well as increase the half-life of the composition. In these preparations the antibiotic is incorporated as part of a liposome as described herein, alone or in conjunction with a targeting molecule which binds to, e.g., a receptor prevalent among the suspected infecting bacterial cells, such as monoclonal antibodies or the binding fragments thereof which bind to the lipid A, core or lipopolysaccharide side chains of gram-negative bacteria, or with other therapeutic or compositions.

A pharmaceutical composition of a liposome/antibiotic suspension prepared according to the present invention can be administered intravenously, locally, topically, etc. in a dose which varies according to, interalia, the manner of administration, the drug being delivered, and the stage of the infection or other condition being treated.

Depending on the intended mode of administration and the intended use, the compositions may be in the form of liquid or semi-solid dosage forms, such, for example, as liquids, suspensions, pastes, creams, etc., and may be in unit-dosage forms suitable for administration of relatively precise dosages. The liposomal/antibiotic compositions may include a conventional pharmaceutical carrier or excipient and, in addition, may include other medicinal agents, antibiotics, growth factors, etc.

For semi-solid compositions, as would be appropriate for pastes and creams intended for topical

administration, the pharmaceutical compositions of the invention can be provided separately or may be compounded with conventional nontoxic carriers. Such compositions may contain about 5-100% active ingredient (liposome/antibiotic complexes), more preferably about 5-25%. The concentration of the complexes in these formulations can vary widely, and will be selected primarily by intended use, viscosities, etc., in accordance with the particular mode of administration selected and the infection being treated. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Science, 17th ed., Mack Publishing Company, Easton, PA (1985), which is incorporated herein by reference. The composition or formulation to be administered will, in any event, contain a quantity of the liposome/antibiotic complexes sufficient to achieve the desired therapeutic or prophilactic effect in the subject being treated.

Preferably, the pharmaceutical compositions are administered parenterally, e.g., intravenously or intramuscularly. Thus, the invention provides pharmaceutical compositions for parenteral administration which comprise a liposomal/antibiotic complex at a physiological osmolarity, suspended in an acceptable carrier as desired, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., 0.4% saline, 0.3% glycine, and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc. Actual methods for preparing

parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Sciences, supra.

The pharmaceutical compositions of the invention are administered to a warm-blooded animal, such as humans, already suffering from an infection in an amount sufficient to terminate or significantly inhibit the progression of the infection. Amounts adequate to accomplish these effects are defined as a "therapeutically effective doses". Amounts effective for this use will depend on the severity of the infection and its site, and the general susceptibility of the bacterium to the antibiotic being used, e.g., netilmicin, and the general state of health of the patient being treated. The amounts wil generally be less than those typically employed with free drug under similar circunstances. The amount of drug administered via the liposomal/drug formulations of the invention can also be increased above those typically used for free drug due to the minimization of toxicity to the patient and the overall increased therapeutic effectiveness of the preparations compared to free drug, as illustrated hereinbelow, as might be necessary in the case of severe, life-threatening infections. Maintenance dosages over a prolonged period of time may be adjusted as necessary. For veterinary uses in animals other than humans higher levels may also be administered as necessary. Determining actual amounts of the liposomal/drug complexes necessary to treat a particular condition as described above will be through standard empirical methods well known in the art.

In prophylactic applications compositions containing the liposomal/drug complexes of the invention are administered to a host susceptible to or otherwise at risk of infection. Such an amount is defined to be a "prophylactically effective dose". In this use, the precise amounts again depend on the host's condition and general

state of health, but generally will be less than those necessary for therapeutic use discussed above. Also, dosages may be less than those typically employed with free drug, particularly in view of the extended half-lives provided by the present formulations. Some prophylactic uses may warrant increased dosages when compared to free drug, but the diminished toxicity and increased therapeutic effectiveness permit higher relative dosages. As mentioned above, liposomes of smaller size, i.e., less than 0.2 μm, appear to be better suited for prophylactic administration than the non-extruded liposomes of 0.8-1 μm due to an increased half-life and other characteristics.

The compositions of the invention, including pharmaceutical compositions, may be administered alone or as adjunct therapy or prophylaxis. The compositions can be used in combination with other drugs, including antibiotics, found to improve treatment responses. In this manner, a synergistic effect may be attained that yields a clinical efficacy greater than that realized with any single factor.

The following examples of chemical and biological analysis made with the liposomal/aminoglycoside formulations prepared by the present processes are offered by way of illustration, not by way of limitation.

EXAMPLES

These examples describe analysis of liposome aminoglycoside formulations prepared as described above, where the aminoglycoside was netilmicin, the lipid mixture was phosphatidylcholine:cholesterol:phosphatidyli-nositol at 5:1:4, with relative lipid concentration of 49-64 mM. Hydration took place with 2.3 to 2.5 mg/ml netilmicin, followed by freezing at -170°C or -70°C and lyophilization.

Rehydration to a final osmolarity of 300 mOSM was accomplished by adding 5% dextrose in one-tenth the volume of antibiotic used, followed by filling to the initial volume with 154 mM NaCl. Upon dilution in saline and extrusion first through a 0.4 μm filter and followed by extrusion through a 0.2 μm polycarbonate membrane and ultracentrifugation at 250,000 x g for 30 min., the final liposomal/aminoglycoside formulation had a diameter of about 0.20 μm ± 0.01 μm.

Characterization of Liposomal Formulation

Table 1 shows encapsulation parameters which characterize the liposomal antibiotic formulations of this invention.

TABLE 1 - Encapsulation Parameters

Liposomal Formulation (Diameter, μm)	Final Drug/Lipid Ratio (mg/100 mg lipid)	Encapsulation Efficiency (%)	Recovery (%)
0.81 - 1.00	0.58 - 0.61	65 - 71	47 - 53
0.19 - 0.21	4.79 - 5.38	82 - 88	61 - 65

Encapsulation efficiency means the ratio of the final drug/lipid and the initial drug/lipid, in percentage. The percent recovery indicates the percentage of final to initial drug concentration. For conversion to moles of lipid, the average molecular weight of lipid is treated as 700 g/mol.

The results obtained show that high recoveries and encapsulation efficiencies are achieved with low drug/lipid ratios for this illustrative liposomal formulation prepared by the present process.

In Vivo Acute Toxicity

The acute toxicity evaluation of both free and liposome encapsulated netilmicin was made in groups of 10 Swiss (Charles River) mice. The animals were injected intravenously with free and liposomal antibiotic in doses of 10 to 50 mg/Kg. The number of surviving animals per dose was determined and the dose which killed 50% of animals (LD_{50}) was calculated (Table 2).

TABLE 2 - Acute Toxicity

Formulation	LD_{50} (mg/Kg)
Free Netilmicin	20 - 28
Liposomal Netilmicin	> 50

The results indicated that formulations prepared by the present process showed a substantial reduction of acute toxicity, since with the maximum possible injected dose 100% of animals survived without any sign of anaphylactic shock.

Pharmacokinetic Studies

Pharmacokinetic studies were done in Swiss (Charles River) mice, normal or granulocytopenic, using

free and liposomal netilmicin. The granulocytopenia was produced by intraperitoneal injections of cyclophosphamide according to standard protocols. The animals were injected intravenously with doses of 4 mg/Kg of the free and liposomal netilmicin preparations and blood was collected at fixed times (Table 3). Netilmicin concentrations were determined by agar disc diffusion assay with B. subtilis as the indicator organism (Anhalt, J.P. (1985), "Assays for Antimicrobial Agents in Body Fluids", in Manual of Clinical Microbiology, ed Lenette, E.H., Chp. 106, pp. 1010-1014, 4th ed., Washington D.C., and Lorian, V. (1986), "Antibiotics in Laboratory Medicine", 2d ed., Williams & Wilkins, Los Angeles).

TABLE 3 - Pharmacokinetics parameters

Animals	Formulation	Half Life Time (min)	Vol.Dist. (SS) (ml)	Mean Residence Time (min)
Normal	Free Netilmicin	12	15.69	21
	Liposomal Netilmicin	138	2.89	204
Granulocy-topenic	Free Netilmicin	11	15.89	19
	Liposomal Netilmicin	219	2.90	330

Vol. Dist. (SS) - Volume of Distribution at the steady state

For liposomal aminoglycoside formulations prepared according to this invention, the half-life circulation time was greatly prolonged when compared to the free drug. The larger increase observed for granulocytopenic animals was probably attributable to their low phagocytic capacity.

Pharmacological Activity

To evaluate the therapeutic efficacy of a liposomal/drug preparation produced according to the present invention, an animal model of peritonitis was perfomed using Swiss (Charles River) mice. The infection was obtained by inoculating with a netilmicin sensitive bacterial suspension of E. coli (approximately $1x10^8$ bacteria/ml). Groups of six mice were used and two types of treatment assayed: prophylactically (given 24 hours before infection) or immediately post-infection (0 hours) (therapeutically). The control group was injected with 154 mM NaCl. The therapeutic dose was 10 fold lower than dose used prophylactically (2 mg/Kg). The percent survival was calculated after 24 days (Table 4).

TABLE 4 - Pharmacological Activity in a Peritonitis Model

	Survival (%)	
Formulation	Prophylactic (-24H)	Therapeutic (OH)
154 mM NaCl	0	0
Free Net.	33	17
Lip. Net. (0.81-1.00 μm)	17	50
Lip. Net. (0.19-0.21 μm)	83	33

Net. - Netilmicin
Lip. - Liposomal

From the results it was concluded that pharmacological activity was greatly increased by liposomal aminoglycoside formulations produced according to the present invention, confirming their usefulness in a clinical setting.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

CLAIMS

1. A process for preparing a liposomal aminoglycoside antibiotic composition suitable for administration to a mammal which comprises:

forming a multilamellar liposome aminoglycoside composition from an aqueous solution of the aminoglycoside and a lipidic film mixture containing a lipid component having a negative charge and which is capable of binding to the aminoglycoside;

lyophilizing the liposomal aminoglycoside;

rehydrating the lyophilized liposomal aminoglycoside to a final physiologic osmolarity, first in a non-saline solution of approximately physiologic osmolarity; and

further rehydrating the liposomal aminoglycoside with a physiologically acceptable saline solution.

2. The process according to claim 1, further comprising the step of sizing the rehydrated liposomal aminoglycoside formulation by extrusion through a porous membrane.

3. The process according to claim 2, wherein the liposomal aminoglycoside formulation is extruded under pressure sequentially through a 0.4 μm and 0.2 μm membrane.

4. The process according to claim 3, wherein the liposomal aminoglycoside is approximately 0.2 μm in diameter.

5. The process according to claim 1, wherein the resulting liposomal aminoglycoside is approximately 0.8 μm - 1 μm in diameter.

6. The process according to claim 3, in which any aminoglycoside not incorporated into the liposomes is

removed before or after the extrusion step.

7. The process according to claim 1, in which the concentration of the lipid mixture in the lipidic film is 30 mM up to 120 mM.

8. The process according to claim 7, in which the concentration of the lipid mixture in the lipidic film is 45 mM up to 65 mM.

9. The process according to claim 1, in which a lipid component of the lipidic film mixture having a negative charge and which is capable of binding to the aminoglycoside is phosphatidylinositol, hydrogenated phosphatidylinositol, phosphatidylinositol mono or biphosphate.

10. The process according to claim 9, in which the concentration of the phosphatidylinositol, hydrogenated phosphatidylinositol, phosphatidylinositol mono or biphosphate is at least 25%.

11. The process according to claim 10, in which the concentration of the phosphatidylinositol, hydrogenated phosphatidylinositol, phosphatidylinositol mono or biphosphate is approximately 40%.

12. The process according to claim 1, in which the lipidic film mixture is comprised of, in a molar ratio ranging from 0.01 to 10: cholesterol (Chol), phosphatidylcholine (PC), distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimirystoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI),

phosphatidic acid (PA), dicetylphosphate (DcP), dimirystoylphosphatidylglycerol (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and any other synthetic lipid.

13. The process of claim 12, in which the lipidic film mixture is comprised of a glycerophospholipid selected from phosphatidylcholine, dimirystoylphosphatidylcholine, dipalmitoylphosphatidylcholine; a cholesterol, opcionally present; and a negatively charged molecule selected from, phosphatidylinositol, hydrogenated phosphatidylinositol, phosphatidylinositol mono and biphosphate or dicetylphosphate, with each component of the liposomal preparation when present in molar ratios of 40-70%, 10-30%, and 20-50%, respectively.

14. The process according to claim 13, in which the lipidic film mixture is comprised of phosphatidylcholine: cholesterol: phosphatidylinositol in molar ratios of 40-70%, 10-30%, and 20-50%, respectively.

15. The process according to claim 14, wherein the lipid components of phosphatidylcholine: cholesterol: phosphatidylinositol are in a relative proportion of approximately 5:1:4.

16. The process according to claim 1, wherein the lyophilized liposomal aminoglycoside is rehydrated in a solution of dextrose, galactose, mannose, sucrose or other sugar at sufficient concentration to maintain a physiologic osmolarity.

17. The process according to claim 1, wherein the physiologically aceptable saline solution of the final rehydration step is NaCl.

18. The process according to claim 1, wherein the aqueous solution of aminoglycoside used to form the multilamellar liposome aminoglycoside composition contains a concentration of aminoglycoside from 2 mg/ml to 50 mg/ml.

19. The process according to claim 1, wherein the aminoglycoside antibiotic is neomycin, kanamycin, amikacin, tobramycin, gentamicin, sisomicin, netilmicin, streptomycin or paromomycin.

20. The process according to claim 19, wherein the aminoglycoside is netilmicin.

21. The process according to claim 18, characterized by producing liposomal netilmicin formulations with an encapsulation efficiency greater than 60%.

22. The process according to claim 21, in which the liposomal netilmicin formulations, when administered to a patient, reduce the acute toxicity (LD_{50}) at least two-fold, compared to free netilmicin.

23. The process according to claim 21, characterized by producing liposomal netilmicin formulations with enhanced therapeutic activity when used in the treatment of a gram-negative infection by a netilmicin-sensitive organism, compared to free netilmicin.

24. A liposomal formulation suitable for administration to a mammal which comprises an aminoglycoside antibiotic characterized by having a drug/lipid ratio up to 6 mg aminoglycoside/100 mg lipid.

25. The liposomal aminoglycoside formulation of claim 24, wherein the liposomes range from 5 μm to 0.01 μm in diameter.

26. The liposomal aminoglycoside formulation of claim 24, wherein the lipid component is comprised of, in a molar ratio ranging from 0.01 to 10: cholesterol (Chol), phosphatidylcholine (PC), distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimirystoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatidic acid (PA), dicetylphosphate (DcP), dimirystoylphosphatidylglycerol (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and any other synthetic lipid.

27. The liposomal aminoglycoside formulation of claim 26, wherein the lipid component is comprised of a glyocerophospholipid selected from phosphatidylcholine, dimiristoylphosphatidylcholine, dipalmitoylphosphatidyl-choline; a cholesterol, optionally present; and a negatively charged molecule selected from phosphatidylinositol, hydrogenated phosphatidylinositol, phosphatidylinositol mono and biphosphate or dicetylphosphate, with each component of the liposomal preparation when present in molar ratios of 40-70%, 10-30%, and 20-50%, respectively.

28. The liposomal aminoglycoside formulation of claim 27, wherein the lipid component is comprised of phosphatidylcholine: cholesterol: phosphatidylinositol in molar ratios of 40-70%, 10-30%, and 20-50%, respectively.

29. The liposomal aminoglycoside formulation of claim 28, wherein the lipid components of phosphatidylcholine: cholesterol: phosphatidylinositol are in a relative proportion of approximately 5:1:4.

30 The liposomal aminoglycoside formulation of claim 28, wherein the phosphatidylinositol is at least 40%.

31. The liposomal aminoglycoside formulation of claim 24, having approximately physiological osmolarity.

32. The liposomal aminoglycoside formulation according to claim 24, in which the aminoglycoside is neomycin, kanamycin, amikacin, tobramycin, gentamicin, sisomicin, netilmicin, streptomycin or paromomycin.

33. The liposomal aminoglycoside formulation according to claim 24, in which the aminoglycoside is netilmicin.

34. The liposomal aminoglycoside formulation according to claims 24 to 33, characterized by an increased plasma half-life time and decreased acute toxicity, compared to free aminoglycoside.

35. The liposomal aminoglycoside formulation according to claims 24 to 33, characterized by having an enhanced therapeutic or prophylactic activity in vivo against aminoglycoside-sensitive bacteria, compared to treatment with free aminoglycoside.

36. A liposomal aminoglycoside formulation according to any of the claims 24 to 33 when prepared by a process according to any of claims 1 to 23.

37. Utilization of liposomal formulations according to claim 36, for treating or preventing an infection in an animal, wherein a therapeutically or prophylactically effective amount of a liposomal aminoglycoside composition prepared by a process according to any of claims 1 to 23,

is used.

38. The utilization according to claim 37, wherein the prophylactic liposome/aminoglycoside formulation comprises liposomes of about 0.2 μm in diameter.

39. The utilization according to claim 37, wherein the therapeutic liposome/aminoglycoside formulation comprises liposomes of about 0.8-1 μm in diameter.

INTERNATIONAL SEARCH REPORT

International Application No PCT/PT 93/00001

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K9/127; A61K31/71

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	EP,A,0 485 143 (LABORATORIO NACIONAL DE ENGENHARIA E TECNOLOGIA INDUSTRIAL) 13 May 1992 see page 2, line 45 - page 3, line 36 see page 5, line 18 - line 32 see claims 1-4,10,11,14,15,20 ---	1-23, 37-39
X	US,A,4 952 405 (YAU-YOUNG) 28 August 1990	24,25, 31,32, 34,35
Y	see column 8 - column 9; example 1 ---	26-30
	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 AUGUST 1993	18. 08. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BENZ K.F.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	CHEMICAL ABSTRACTS, vol. 107, no. 13, 28 September 1987, Columbus, Ohio, US; abstract no. 108910p, S. AU ET AL 'aminoglycoside antibiotics preferentially increase permeability in phosphoinositide-containing membranes: a study with carboxyfluorescein in liposomes' page 25 ;column 1 ; see abstract & BIOCHIM. BIOPHYS. ACTA vol. 902, no. 01, 1987, pages 80 - 86 ---	10,11, 13-15, 26-30
Y	WO,A,8 804 573 (THE LIPOSOME COMPANY, INC) 30 June 1988 cited in the application see the whole document ---	1-8,12, 16-19, 37-39
Y	WO,A,8 505 030 (THE LIPOSOME COMPANY) 21 November 1985 see page 14, line 10 - page 23, line 13 see page 16, line 25 - page 17, line 6 see claim 92 & US,A,4 897 384 cited in the application ---	9,20-23
A	JOURNAL OF LIPOSOME RESEARCH vol. 2, no. 1, April 1992, pages 11 - 22 , XP246007 Y. CAJAL ET AL. 'gentamicin encapsulation in liposomes: factors affecting the efficiency' -----	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/PT 93/ 00001

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: Although claims 37-39 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.